pH Dependence of the Tryptophan Fluorescence in Cytochrome *c* Oxidase: Further Evidence for a Redox-Linked Conformational Change Associated with Cu_A[†]

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ABSTRACT: When the low-potential metal centers of cytochrome *c* oxidase are reduced, the enzyme undergoes a conformational transition that shifts the fluorescence maximum of the emitting tryptophan residues from 329 to 345 nm. At pH 7.4, the change in this tryptophan fluorescence intensity is a nonlinear function of the electron equivalents added to the cyanide-inhibited enzyme. This nonlinear behavior is a result of the difference in redox potential between cytochrome *a* and Cu_A, which, at equilibrium, favors electron occupancy at cytochrome *a*. Studies on the cyanide-inhibited enzyme suggest that the conformational change is associated with reduction of Cu_A [Copeland, R. A., Smith, P. A., & Chan, S. I. (1987) *Biochemistry 26*, 7311–7316]. In this work we present tryptophan fluorescence data for the cyanide-inhibited enzyme at pH 8.9. Because of the pH dependence of the midpoint potential of cytochrome *a* in this form of the enzyme, the two low-potential centers become virtually isopotential at pH 8.9. The results obtained confirm our earlier conclusion that the observed conformational change is linked to the reduction of Cu_A only, rather than to the redox activity of both low-potential metal centers. We find that, in partially reduced cyanide-inhibited oxidase, raising the pH from 7.4 to 8.9 results in an intensification and red shift of the enzyme's tryptophan emission as the electron occupancy redistributes from cytochrome *a* to Cu_A. Moreover, when the fluorescence change is plotted as a function of the number of electrons added to the enzyme at pH 8.9, the data fit the nearly linear function expected for a conformational change triggered by reduction of Cu_A exclusively.

Cytochrome *c* oxidase,[1] the terminal enzyme in the respiratory electron-transfer chains of mitochondria and several aerobic bacteria, catalyzes the four-electron reduction of dioxygen to water (Wikström et al., 1981). The enzyme contains four redox-active metal cofactors: two heme A chromophores (cytochrome *a* and cytochrome a_3) and two copper ions (Cu_A and Cu_B). Cytochrome *a* and Cu_A serve as the initial electron acceptors from ferrocytochrome *c*, while cytochrome a_3 and Cu_B together form the site of dioxygen binding and reduction. At some point in the electron-transfer reaction, protons are actively pumped across the inner mitochondrial membrane in which the enzyme resides. It is now established that redox activity at one or both of the low-potential metal centers, cytochrome *a* and Cu_A, is linked to the electron-coupled proton translocation (Wikström & Casey, 1985). The nature of the energetic link between redox activity and proton pumping is poorly understood at present, although several theoretical models for this coupling have been proposed (Gelles et al., 1986; Wikström et al., 1981; and references cited therein). However, it is becoming increasingly clear that some type of communication between the metal center(s) and the protein matrix is needed to accomplish proton pumping. Models have been put forth in which both localized structural changes (i.e., metal–ligand rearrangements) and/or large-scale protein conformational changes participate in the redox-linked proton translocation [see Gelles et al. (1986) for a review].

In a recent series of papers, this laboratory has established that a conformational transition occurs upon electron input to the low-potential metal centers of cytochrome *c* oxidase. This conformational transition can be conveniently monitored by tryptophan fluorescence spectroscopy (Copeland et al., 1987; Nilsson et al., 1988b). On the basis of a detailed analysis of the electron-titration fluorescence data (Copeland et al., 1987) and the effects of thermal modification of the Cu_A site (Nilsson et al., 1988b), it was argued that the redox activity at Cu_A is a necessary element for the triggering of this conformational change. Some ambiguity remains, however, as to whether this conformational transition is the outcome of the reduction of Cu_A exclusively or of the reduction of both low-potential sites.

In the present work we have exploited the −30 mV/pH unit dependence of the cytochrome *a* midpoint potential in the cyanide-inhibited enzyme (Artzabanov et al., 1978; Ellis, 1986) to settle this issue. The difference in midpoint potential between cytochrome *a* and Cu_A ($\Delta E^{\circ\prime}_{cyta\text{-}Cu_A}$) decreases from +48 to +3 mV in the cyanide-inhibited oxidase (in which both cytochrome a_3 and Cu_B are locked into their oxidized states) between pH 7.4 and 8.9. If an electron equivalent is added to this form of the enzyme, the distribution of the electrons between cytochrome *a* and Cu_A is expected to be pH dependent. In particular, raising the pH from 7.4 to 8.9 should result in a significant redistribution of electron occupancy in favor of Cu_A. Accordingly, we have (i) monitored the tryptophan fluorescence spectrum of a ca. one-electron-reduced,

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[1] Abbreviations: cytochrome *c* oxidase, ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1); D, deuterium ion; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tween 20, poly(oxyethylene)-sorbitan monolaurate; UV–vis, ultraviolet–visible.

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cyanide-inhibited cytochrome *c* oxidase as a function of pH and (ii) repeated the electron-titration experiment of Copeland et al. (1987) at pH 8.9. The results of both experiments confirm our earlier hypothesis that the conformational transition monitored by tryptophan emission is linked exclusively to redox events at the Cu_A site.

Materials and Methods

Materials. Cytochrome *c* oxidase was isolated from bovine heart by the method of Hartzell and Beinert (1974). The enzyme was solubilized in 0.05 M Hepes buffer (pH 7.4) containing 0.5% Tween 20 (Sigma) and stored at −80 °C until just prior to use. The cyanide-inhibited enzyme was prepared by adding aqueous KCN to a ca. 300 μM solution of resting state cytochrome *c* oxidase to a final concentration of 5 mM in CN^- and incubating for 48 h at 4 °C. The extent of cyanide binding to cytochrome a_3 was followed spectrophotometrically by observing the shift of the enzyme's Soret absorption maximum from 418 to 427 nm. Final concentrations of the cyanide-inhibited enzyme were determined spectrophotometrically with an extinction coefficient of 156 mM^{-1} cm^{-1} at 427 nm (Blair et al., 1982). Samples for fluorescence spectroscopy were prepared by diluting the stock solution of cyanide-inhibited enzyme into 0.05 M Hepes buffer (pH 7.4 or 8.9) containing 0.167 M K_2SO_4, and 0.5% lauryl maltoside (Calbiochem) to a concentration of 2–3.6 μM.

Ferrocytochrome *c* (Sigma type VI) was prepared as described in Copeland et al. (1987). The cytochrome *c* was stored as a concentrated (ca. 4 mM) stock solution at −80 °C until just prior to use. Solutions of 150 μM ferrocytochrome *c*, at pH 7.4 or 8.9, were prepared in the same buffer system as for the cytochrome *c* oxidase (vide supra) by dilution of the stock ferrocytochrome *c*. The final concentration of ferrocytochrome *c* solutions was determined spectrophotometrically with an extinction coefficient of 29.5 mM^{-1} cm^{-1} at 550 nm (Sigma Chemical Co. Catalog, 1986).

Methods. In the fluorescence experiments, a known volume of 150 μM cytochrome *c* solution at the appropriate pH was delivered into 3 mL of cytochrome *c* oxidase solution with a 25-μL Hamilton syringe. The number of electrons actually transferred from cytochrome *c* to the oxidase was determined from the diminution of the ferrocytochrome *c* absorption band at 550 nm with a change in extinction coefficient of 21.0 mM^{-1} cm^{-1} upon oxidation (Wikström & Sigel, 1979).

Fluorescence spectra were recorded on a SLM 4800 spectrofluorometer equipped with a SMC-210 monochromator controller and SE-480–485 electronics (SLM Instruments), which was interfaced to an IBM XT computer. All the reported fluorescence data have been corrected for instrument response, enzyme concentration, and inner filter effects as previously described (Copeland et al., 1987).

UV–vis absorption spectra (700–220 nm) were recorded for each sample with a Beckman DU-7 HS scanning spectrophotometer immediately before fluorescence spectral acquisition.

Theoretical electron occupancies of the low-potential metal centers were determined as in Copeland et al. (1987). For a given level of reduction of the cyanide-inhibited oxidase, *N*, the populations of enzyme with both metal centers oxidized, with only Cu_A reduced, with only cytochrome *a* reduced, and with both metal centers reduced (represented by n_O, n_{Cu_A}, n_{cyta}, and n_R, respectively) were calculated by using two parameters: (i) the difference in redox potential between the two metal centers, $\Delta E^{\circ\prime}_{cyta\text{-}Cu_A}$, and (ii) the anticooperative redox interaction in the reduction of the two metal centers, $\Delta E^{\circ\prime}_{cooperative}$. Literature values of $\Delta E^{\circ\prime}_{cyta\text{-}Cu_A}$ = 48 mV at pH 7.4 (Goodman, 1984), $\Delta E^{\circ\prime}_{cyta\text{-}Cu_A}$ = 3 mV at pH 8.9 (Artzatbanov, 1978), and $\Delta E^{\circ\prime}_{cooperative}$ = −40 mV (Wang, 1986; Ellis, 1986) were used in the analysis. The results of the analysis were insensitive to $\Delta E^{\circ\prime}_{cooperative}$.

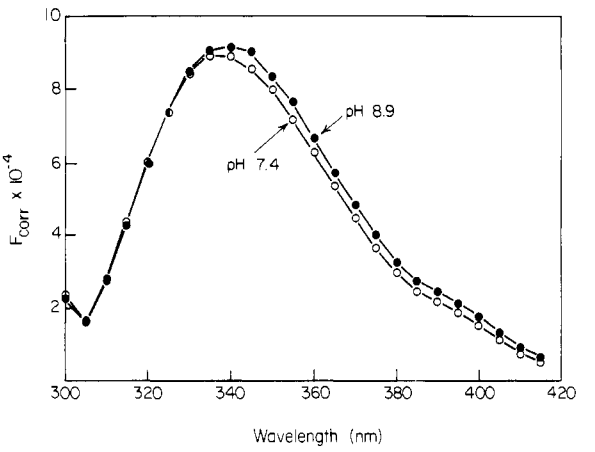

FIGURE 1: Corrected fluorescence spectra of cyanide-inhibited cytochrome *c* oxidase reduced by 1.28 equiv from ferrocytochrome *c* at pH 7.4 (○) and pH 8.9 (●). Each spectrum represents the sum of four consecutive scans.

Table I: Predicted and Observed Percent Fluorescence Change at 355 nm upon Raising the pH from 7.4 to 8.9 for Partially Reduced Cyanide-Inhibited Cytochrome *c* Oxidase[a]

	sample 1	sample 2
electron equiv in cytochrome *c* oxidase	1.23	1.28
theoretical change (%) for a two-electron process at Cu_A and cytochrome *a*	+1.4	+1.0
theoretical change (%) for a one-electron process at cytochrome *a*	−8.1	−7.7
theoretical change (%) for a one-electron process at Cu_A	+10.0	+9.2
experimentally measured change (%)	+8	+7

[a] Further details are in the text.

Results and Discussion

Figure 1 shows the effects of increasing pH on the tryptophan emission spectrum of a sample of cyanide-inhibited cytochrome *c* oxidase that has been reduced by 1.28 electron equiv from ferrocytochrome *c*. At pH 7.4 the emission maximum is at 334 nm, and the band is somewhat broadened relative to the spectrum in the absence of added electrons. Raising the pH from 7.4 to 8.9 has two effects on the spectrum: (i) there is a red shift of the emission maximum and (ii) an overall increase in emission intensity is also observed. Both the red shift and the enhancement in emission are similar in direction (but different in magnitude) to those previously reported for complete reduction of the native enzyme at pH 7.4 (Copeland et al., 1987). The fluorescence difference spectrum between the pH 8.9 and pH 7.4, partially reduced, cyanide-inhibited oxidase exhibits a maximum at 355 nm, as does the difference spectrum of the fully reduced minus fully oxidized protein. Control studies in which the pH of the enzyme solution is varied over the range 6.5–9.1 in the absence of added electron equivalents show no changes in the tryptophan fluorescence spectrum relative to pH 7.4. Moreover, no significant change in the overall level of reduction of cytochrome *c* oxidase by ferrocytochrome *c* is observed when the pH is raised. Thus the observed fluorescence changes appear to be associated with electron redistribution between cytochrome *a* and Cu_A.

The change in tryptophan fluorescence at 355 nm with pH is summarized in Table I for two samples of partially reduced,

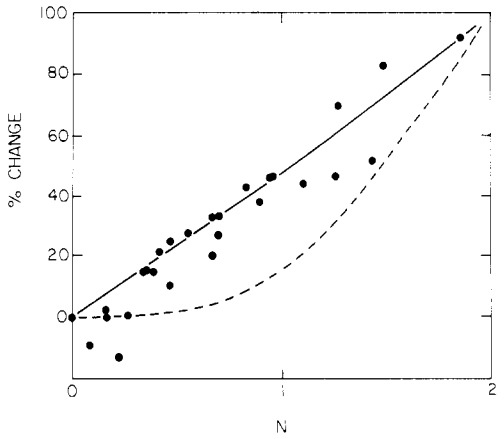

FIGURE 2: Plot of percent fluorescence change at 355 nm vs electron equivalents accepted (N) from ferrocytochrome c by cyanide-inhibited cytochrome c oxidase at pH 8.9. Each data point represents the average value of three measurements. The maximum standard deviation for any data point was ±8% of the mean value. The solid line is the theoretically predicted behavior for a one-electron process at Cu_A, while the dashed line is the theoretically predicted behavior for a two-electron process at cytochrome a and Cu_A. Further details are in the text.

cyanide-inhibited oxidase. Also shown for comparison are the theoretically predicted values of the fluorescence change if the process were assumed to be dependent on Cu_A reduction, on cytochrome a reduction, and on reduction of both metal centers. The change in fluorescence is defined by

$$\% \text{ change} = 100(F_{8.9} - F_{7.4})/F_{7.4} \quad (1)$$

where $F_{8.9}$ is the corrected fluorescence at pH 8.9 and $F_{7.4}$ is the corrected fluorescence at pH 7.4. The theoretical values for the fluorescence at pH 7.4 and pH 8.9 are calculated by the equation

$$F = n_O F_O + n_{Cu_A} F_{Cu_A} + n_{cyta} F_{cyta} + n_R F_R \quad (2)$$

where n_i is the theoretically calculated population of the enzyme in state i and F_i is the fluorescence that would be measured if the enzyme were entirely in state i. The experimentally determined change in the tryptophan fluorescence at 355 nm upon complete reduction of cytochrome c oxidase is +50%; therefore, we used (i) $F_R = F_{Cu_A} = 1.5F_{cyta} = 1.5F_O$ for a process dependent exclusively on Cu_A, (ii) $F_R = 1.5F_{Cu_A} = 1.5F_{cyta} = 1.5F_O$ for a two-electron process, and (iii) $F_R = 1.5F_{Cu_A} = F_{cyta} = 1.5F_O$ for a cytochrome a dependent process. The experimental values clearly support the hypothesis of a one-electron process at Cu_A.

As noted by Scholes and Malmström (1986), if only one of two isopotential metal centers is responsible for a redox-linked conformational change, the extent of conformational change observed should be proportional to the number of electrons introduced into the protein, even in the presence of an anticooperative interaction between the two metal centers. Since $\Delta E^{\circ\prime}_{cyta\text{-}Cu_A}$ of the cyanide-inhibited enzyme is only 3 mV at pH 8.9, one expects the dependence of the tryptophan fluorescence change on the number of added electron equivalents to be close to linear, if the conformational change monitored by tryptophan emission is dependent on Cu_A reduction only. The solid line in Figure 2 represents the expected behavior for the one-electron process at Cu_A, while the dashed curve represents the expected behavior for a two-electron process that is dependent on the reduction of both Cu_A and cytochrome a. Also presented in Figure 2 for comparison with the theoretical predictions are the measured changes in fluorescence intensity at 355 nm with varying numbers of electron equivalents in the cyanide-inhibited enzyme at pH 8.9. The percentage change in fluorescence reported here is given by

$$\Delta F = 100(F_N - F_O)/(F_\infty - F_O) \quad (3)$$

where F_N is the measured corrected fluorescence upon the addition of N electron equivalents to cytochrome c oxidase, F_O is the corrected fluorescence of the fully oxidized protein, and F_∞ is the corrected fluorescence of the CN^--mixed valence oxidase reduced with a slight excess of sodium dithionite. Although there is some scatter in the experimental data points, the observed titration behavior is clearly best modeled by a one-electron process at Cu_A. When the data are subjected to least-squares analysis for linear, polynomial, and exponential functions, the linear function offers the best fit and closely approximates the theoretically predicted behavior for a Cu_A-dependent process.

The data presented here provide clear evidence that the conformational transition monitored by tryptophan fluorescence is linked to reduction of the Cu_A site exclusively. This conclusion is consistent with our previous analysis of the electron-titration tryptophan fluorescence data at pH 7.4; it is also in accordance with the effects of disruption of the Cu_A site on the tryptophan fluorescence recently reported on the heat-treated enzyme (Nilsson et al., 1988b).

The question of whether this conformational transition is coupled to redox activity at one or two metal centers becomes important when models of proton pumping that include obligatory conformational changes in the mechanism of translocation are considered. Most models for redox-linked proton pumping by cytochrome c oxidase rely on redox activity at a single metal center, either cytochrome a or Cu_A, to provide the driving force for proton translocation. Wikström and co-workers have developed a protein conformation-dependent model of proton pumping, where they argue in favor of cytochrome a as the site of redox coupling (Wikström, 1978). The most intriguing experimental evidence in favor of cytochrome a as the site of redox coupling has recently been provided by Babcock and co-workers, who showed that the rate of H/D exchange for the protein hydrogen that hydrogen bonds to the cytochrome a formyl oxygen is accelerated by enzyme turnover (Babcock et al., personal communication). An alternative model has been put forth by Chan and co-workers, on the basis of localized conformational transitions in the vicinity of the Cu_A site (Gelles et al., 1986). Several experimental results support a role for Cu_A in the proton pumping mechanism. Nilsson et al. (1988a) have shown that when Cu_A is modified by p-(hydroxymercuri)benzoate, the enzyme no longer sustains proton pumping when reconstituted into phospholipid vesicles; the vesicles containing the modified enzyme become atypically permeable to protons. Cytochrome c oxidase in which Cu_A has been thermally modified likewise shows this unusual proton permeability when reconstituted into vesicles (Li et al., 1988; Nilsson et al., 1988b). These authors argue that the increased proton permeability of the modified Cu_A vesicles results from disruption of the proton gate, creating a passive proton channel through the enzyme.

At present, we have no experimental evidence to directly associate the conformational change monitored by tryptophan fluorescence with the enzyme's proton pumping mechanism. Experiments to address this issue are currently in progress. If a role for this conformational change can be established in the mechanism of proton pumping, it would certainly be compelling evidence for Cu_A as the site of redox-linked proton translocation.

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REFERENCES

Artzatbanov, V. Y., Konstantinov, A. A., & Skulachev, V. P. (1978) *FEBS Lett. 87*, 180–185.

Blair, D. F., Bocian, D. F., Babcock, G. T., & Chan, S. I. (1982) *Biochemistry 21*, 6928–6935.

Copeland, R. A., Smith, P. A., & Chan, S. I. (1987) *Biochemistry 26*, 7311–7316.

Ellis, W. R., Jr. (1986) Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

Gelles, J., Blair, D. F., & Chan, S. I. (1986) *Biochim. Biophys. Acta 853*, 205–236.

Goodman, G. (1984) *J. Biol. Chem. 259*, 15094–15099.

Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318–338.

Nilsson, T., Gelles, J., Li, P. M., & Chan, S. I. (1988a) *Biochemistry 27*, 296–301.

Nilsson, T., Copeland, R. A., Smith, P. A., & Chan, S. I. (1988b) *Biochemistry* (submitted for publication).

Scholes, C. P., & Malmström, B. G. (1986) *FEBS Lett. 198*, 125–129.

Wang, H., Blair, D. F., Ellis, W. R., Jr., Gray, H. B., & Chan, S. I. (1986) *Biochemistry 25*, 167–171.

Wikström, M. (1978) in *The Proton and Calcium Pumps* (Azzone, G. F., et al., Eds.) pp 215–226, Elsevier/North-Holland, New York.

Wikström, M., & Sigel, E. (1979) in *Membrane Biochemistry* (Carafoli, E., & Semenza, G., Eds.) pp 82–91, Springer-Verlag, New York.

Wikström, M., & Casey, R. P. (1985) *J. Inorg. Biochem. 23*, 327–334.

Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase, A Synthesis*, Academic, New York.

Stabilization of Microtubules by Inorganic Phosphate and Its Structural Analogues, the Fluoride Complexes of Aluminum and Beryllium†

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ABSTRACT: In order to elucidate how the elementary reactions of GTP cleavage and subsequent inorganic phosphate (P_i) release, which accompany microtubule assembly, regulate microtubule dynamics, the effect of P_i and of its structural analogues AlF_4^- and BeF_3^- on the stability of GDP–microtubules has been investigated. Inorganic phosphate binds to microtubules with a low affinity (K_D = 25 mM) and slows down the rate of GDP–subunit dissociation by about 2 orders of magnitude. AlF_4^- and BeF_3^- exhibit phosphate-like effects with 1000-fold higher affinity. Evidence has been obtained for direct binding of BeF_3^- to microtubules with a stoichiometry of 1 mol of BeF_3^- per mole of GDP–subunit and an equilibrium dissociation constant of 12–15 μM. AlF_4^- and P_i compete for this site. Phosphate analogues abolish oscillatory polymerization kinetics and slow down microtubule turnover at steady state. In view of these results, we propose that P_i and its structural analogues bind to the site of the γ-phosphate of GTP in the E site and reconstitute a GDP–P_i–microtubule, from which tubulin subunits dissociate very slowly. We therefore understand that, following GTP cleavage on microtubules, P_i release in the medium is accompanied by a structural change resulting in a large destabilization of the polymer. A cap of slowly dissociating GDP–P_i–subunits prevents depolymerization of the microtubule GDP–core at steady state. The similarity with the actin system [Carlier, M.-F., & Pantaloni, D. (1988) *J. Biol. Chem. 263*, 817–825] is underlined.

Microtubules behave in a paradoxical fashion with regard to classical thermodynamics. While systems generally evolve toward a greater stability, microtubules form from GTP–tubulin, GTP is hydrolyzed following the incorporation of tubulin, P_i[1] is released into the medium (Weisenberg et al., 1976; Carlier & Pantaloni, 1981), and the resulting GDP–microtubules are unstable and rapidly lose subunits upon dilution; in contrast, in a regime of growth, the dissociation rate constant of terminal subunits is very low (Hill & Carlier, 1983; Carlier et al., 1984). The loss and rebuilding of a putative GTP–cap has been, to date, the only proposed (Mitchison & Kirschner, 1984) and successfully modeled (Chen & Hill, 1985) hypothesis accounting for the reported dynamic instability features of microtubules (Horio & Hotani, 1986; Cassimeris et al., 1986; Sammak et al., 1987). If some of the kinetics (Johnson & Borisy, 1977) of microtubule assembly are well accounted for by models of reversible polymerization, the dynamic instability behavior is most likely explained by the absence of microreversibility, due to the involvement of GTP hydrolysis, in some step of the assembly process.

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[1] Abbreviations: P_i, inorganic phosphate; MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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